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In vitro Studies

Mechanisms implicated in the effects of boron on wound healing

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Abstract

Recently, we demonstrated that boron modulates the turnover of the extracellular matrix and increases TNFα release. In the present study, we used an *in vitro* test to investigate the direct effect of boron on specific enzymes (elastase, trypsin-like enzymes, collagenase and alkaline phosphatase) implicated in extracellular matrix turnover. Boron decreased the elastase and alkaline phosphatase activity, but had no effect on trypsin and collagenase activities. The effect of boron on the enzyme activities was also tested in fibroblasts considered as an *in vivo* test. In contrast to the results obtained *in vitro*, boron enhanced the trypsin-like, collagenase, and cathepsin D activities in fibroblasts. Boron did not modify the generation of free radicals compared to the control and did not seem to act on the intracellular alkaline phosphatase activity, However, as it did enhance phosphorylation, it can be hypothesized that boron may affect living cells via a mediator, which could be TNFα whose transduction signal involves a cascade of phosphorylations.

Key words: boron, wound healing, TNFα, protease activities, fibroblasts

Introduction

Many studies have been conducted on compounds that would accelerate the healing process. The huge number of products being recommended indicates that the problem is yet to be solved.

Boron has been shown to be beneficial in wound healing: application of a 3% boric acid solution greatly improves the healing of deep wounds, reducing by two thirds the time required in intensive care (1, 2). This has led us to investigate the properties of boron, which, in the past, was known in the pharmacopeia (boric water) as an antimicrobial agent. The use of boron has gradually declined due to the side effects observed after ingestion (3, 4), although facial wounds are still routinely treated with 10% boric petroleum jelly, and Sobel and Chain (5) mentioned the successful topical application of boric acid (600 mg/day for 14 days) for recurrent *Candida glabrata vaginitis*. More recently, Benderdour et al. (6, 7) showed that boron affects the synthesis of the extracellular matrix (ECM), which plays an important role in the wound repair process by increasing the release of proteoglycans, collagen and proteins. It also stimulates the synthesis and release of tumor necrosis factor (TNFα).

The purpose of the present study was to determine whether boron directly affects the ECM turnover in human fibroblasts or acts via an indirect mechanism by influencing the generation of reactive oxygen species (ROS) or the release of cytokines such as TNFα. *In vitro* (direct effect) and *in vivo* (human fibroblasts) tests were performed to investigate the influence of boron on specific proteases (collagenase, trypsin-like, elastase, cathepsin D) or alkaline phosphatase (AP) known to play an important role in the wound healing process, protein phosphorylation and free radical generation.

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Materials and methods

Effect of boron on enzyme activities in vitro

Measurement of the direct effect of boron on protease activity

The activities of 3 enzymes were assayed: elastase, collagenase, and trypsin-like enzyme.

Elastase: 50 µL elastase solution (Calbiochem, La Jolla, CA, USA) containing 0–0.06 U were added to 50 µL (0.24 mmol/L) substrate (AAPV-Rho 110, Cell Probe, Meudon, France), the volume was brought to 2 mL with Tris-HCl pH = 6.62 supplemented with 0–50 mmol/L boric acid. The mixture was incubated for 15 min at 37 °C. The fluorescence was read at λexc 488 nm, λem 525 nm with a spectrofluorometer (Hitachi, ScienceTec, Les Ulis, France).

Collagenase (bacterial origin): 50 µL collagenase solution (Sigma, St. Louis, MO, USA) containing 0–10 µg enzyme were added to 100 µL substrate (GPLGP-Rho 110, Cell Probe). The final volume was adjusted to 2 mL with Tris-HCl pH = 7.6 containing 0–50 mmol/L boric acid. The mixture was incubated for 15 min at 37 °C. The fluorescence was read: λexc 488 nm, λem 525 nm.

The trypsin-like activity was measured at pH = 7.8 with the universal protease substrate (Boehringer, Mannheim, Germany) according to the recommendations of the manufacturer using 2.5% porcine trypsin solution (Boehringer) as enzyme source and incubation for 30 min at 37 °C. The fluorescence was read: λexc 574 nm, λem 584 nm.

Effect of boron on enzyme activities in vivo

Protease activity in fibroblasts

Human fibroblasts (46BR-IG, transformed lung fibroblasts from the European collection of animal cell culture) ($4 \cdot 10^6$ cells) were cultured for 6 h in minimun essential medium (MEM; Gibco, France) with or without boric acid (concentrations given in the results section). A part of the cells was collected by centrifugation at 800 g for 5 min, washed with 0.15 mol/L NaCl and lysed in 50 mmol/L Tris pH = 7.4, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1mmol/L EDTA. The solutions were cleaned by centrifugation and the supernatants were stored at – 20 °C, to be used for the total protease activity measurement. The other part of the cells was trypsinized (0.25% trypsin, Boehringer) for 5 min and centrifuged at 800 g for 5 min. The pellet was washed two times with PBS and suspended in PBS (10^6 cells/mL). Aliquots (50 µL) of the cell suspension were incubated at 37 °C for 5 min, and then 25 µL (0.08 mol/L) cathepsin D substrate VK-Rho 110, or 25 µl (0.08 mol/L) collagenase substrate GPLGGP-Rho 110 (Cell Probe) were added and the mixtures were incubated for exactly 10 min at 37 °C. The reaction was stopped by placing the tubes in ice and by adding 1 mL iced PBS. 25 000 cells were analysed by flow cytometry (Epics cytometer, Coultronic, Margency, France) equipped with an argon laser (λexc 488 nm). The fluorescence was displayed on a monoparametric histogram (256 channels logarithmic scale) and was expressed as mean intensity of fluorescence (MIF). $MIF = e^{[\ln 1000/256]\, x}$, where x is the mean peak channel on the logarithmic scale.

Measurement of the rate of protein phosphorylation

Measurement of direct boron effect on alkaline phosphatase activity

Human serum (1 mL) was mixed with 200 µL 0–50 mmol/L boric acid. The AP activity was measured with a Randox kit (Randox, Roissy, France) with p-nitrophenyl phosphate as substrate and using an automatic apparatus (Wako, Biochem, France). The detection limit of the method was 2 UI/L.

Protein phosphorylation

The protein phosphorylation was measured in fibroblasts cultured for 1 h, 2 h, 4 h, and 6 h in MEM with or without 25 mmol/L boric acid. 0.37 MBq/mL λ [^{33}P]-ATP (111 TBq/mmol, NEN, Boston, USA) and 100 µL ATP (30 mg/mL) were added to the culture medium. The reaction was stopped by adding 200 µL 0.5 mol/L ATP/0.1 mol/L formic acid. The cells were trypsinized, lysed, and the proteins were precipitated with 5% TCA and collected by centrifugation. They were dissolved in 200 µL 0.4% desoxycholate/0.1 mol/L NaOH. The radioactivity was counted and the results are expressed as cpm/10^6 cells.

Measurement of radical oxygen species (ROS)

Two series of experiments were carried out. In the first experiment, the fibroblasts were cultured for 6 h with increasing amounts of boric acid and collected as described below. The cells were suspended in 1 mL MEM containing 10 µL dihydrorhodamine 123 (DRH 123; 0.1 mol/L in dimethylformamide) for 5 min at 37 °C, or in 1 mL MEM containing 20 µL dichlorofluorescein diacetate (DCFH-DA; 0.5 mol/L in dimethylformamide) for 20 min at 37 °C. In the second experiment, the kinetic of free radical generation was evaluated without boric acid or with 0.25% boric acid. The reactions were stopped by placing the tubes in the ice bath. 25 000 cells were analyzed by flow cytometry.

Results

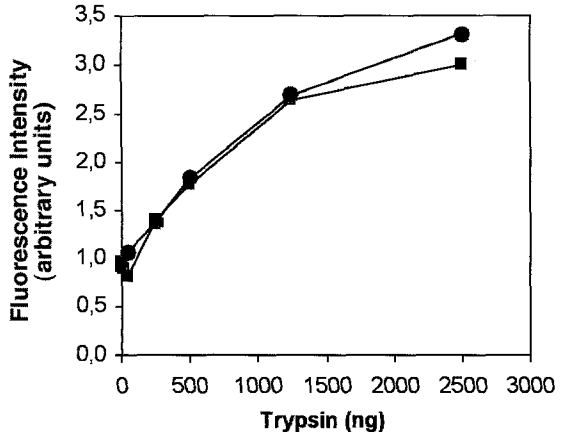
Effect of boron on proteases in vitro

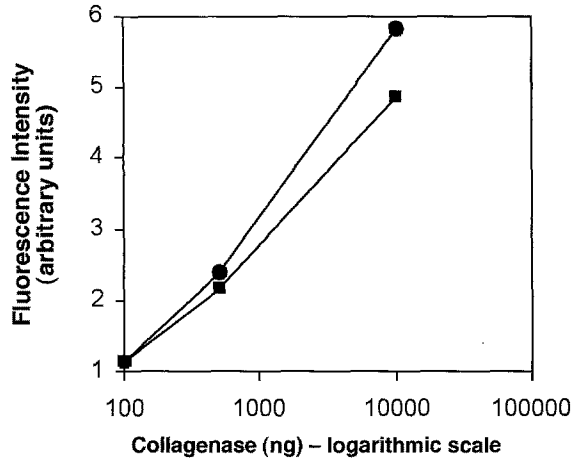
Boron was tested at concentrations of 0–50 mmol/L. Only the results obtained with 25 mmol/L boric acid and different concentrations of enzymes are shown (Fig. 1). Boric acid added to the reaction mixture did not change the trypsin-like or collagenase activity but it inhibited the elastase activity.

Effect of boron on proteases in vivo

The total protease activity was measured in cell lysates. Boric acid (25 mmol/L) significantly ($p < 0.001$) increased the trypsin-like activity. The activity in controls was 38 ± 9 ng equivalent trypsin/10^7 cells while that of the cells treated with boric acid was 79 ± 11 ng/10^7 cells ($n = 6$).

The activities of collagenase and cathepsin D were measured in viable cells by flow cytometry. Boric acid, tested at concentrations of 10–50 mmol/L, enhanced the cathepsin D and collagenase activities in cells; the maximun





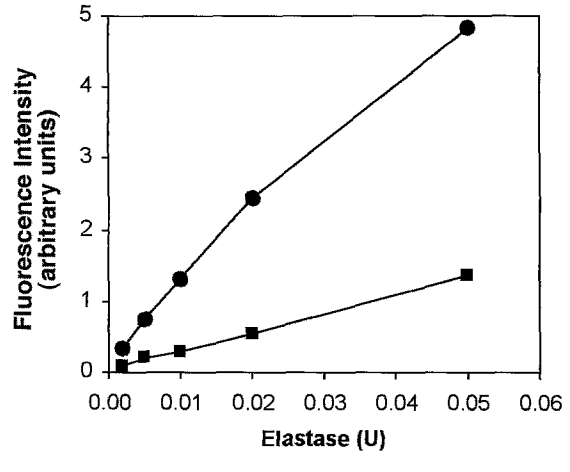


Fig. 1. Direct effect of boric acid on the activities of purified trypsin, elastase, and collagenase. The results are the mean of 4 assays; enzyme activities were measured using specific fluorescent substrates; ■—■ control without boric acid, ●—● assay with 25 mmol/L boric acid.

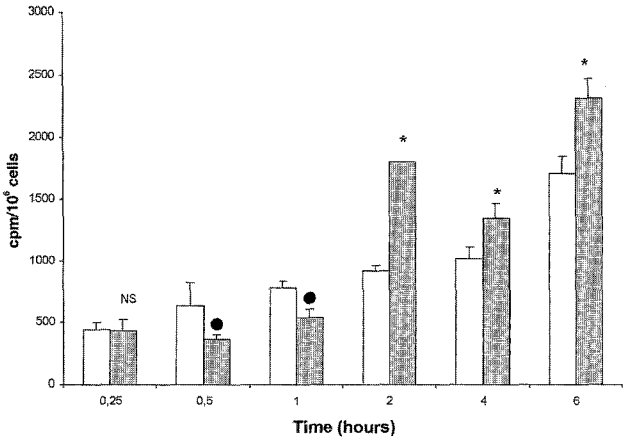
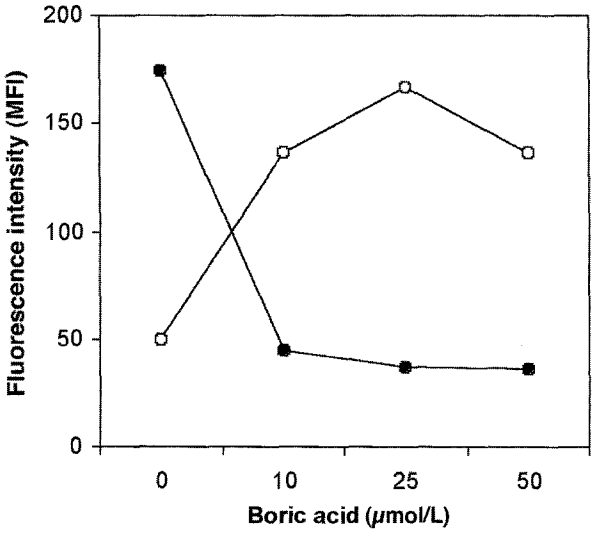


Fig. 2. Protein phosphorylation in fibroblasts incubated wihout boric acid (□) and with 25 mmol/L boric acid (▒). The results were compared by Student t-test. The results are the mean of 4 replicates. * $p < 0.05$ (increased phosphorylation), • $p < 0.05$ (decreased phosphorylation), NS not significant.



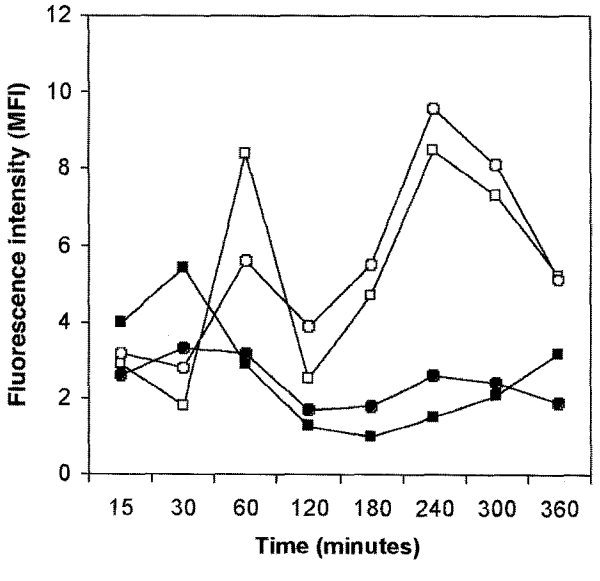


Fig. 3. Measurement by flow cytometry of free radicals in fibroblasts, (**A**) as a function of boric acid concentration; ○—○DCFH, ●—●DHR; (**B**) as a function of time; □—□control, ○—○assay (DCFH); ■—■control, ●—●assay (DHR). ▶

effect was obtained at 25 mmol/L of boric acid. The MFI increased from 60.6 to 90.3 (cathepsin D), and from 69.8 to 89.5 (collagenase).

Phosphatase activity and protein phosphorylation

Boron decreased the alkaline phosphatase activity of human serum (direct effect) from 247 ± 5 to 243 ± 3, 239 ± 5, 231 ± 5, 224 ± 7 UI/mL for 0, 5, 10, 25, 50 mmol/L boric acid (n = 7 for each assay). The decrease was significant from 25mmol/L. The amount of phosphorylated proteins increased with the time in culture, from two hours there was a significant difference between the [^{33}P]-ATP incorporated into the proteins of the control and of the assay (Fig. 2).

Measurement of ROS (free radicals)

Incubating fibroblasts for 6 h with increasing concentrations of boric acid caused a decrease in the fluorescence of DHR 123 (probe for the superoxide anion) and a parallel increase in the fluorescence of DCFH (probe for H_2O_2 and hydroperoxides) (Fig. 3A). However, 25 and 50 mmol/L boron did not stimulate the superoxide dismutase (SOD) activity of human erythrocytes, as measured with the Ransod kit (Randox, Creemlin, UK) (data not shown).

The rate of formation of intracellular ROS was also measured with 25 mmol/L boric acid in function of the culture time. The concentration of H_2O_2 changed with the incubation time, and there were two peaks, one observed at 1 h and the other at 4 h. The concentration of $O_2^{\bullet 2-}$ increased at the beginning of the experiment, but decreased thereafter. However, the results of the controls were not significantly different from those of the assays (Fig. 3B).

Discussion and conclusion

The mechanism by which boron acts in wound healing is unclear, although some studies have shown that it is involved in proteoglycan, collagen and protein synthesis (6, 7). In this study, we measured the direct effect of boron on specific enzymes involved in extracellular matrix turnover and metabolism, and its effect on these enzymes in living cells. Boron directly inhibited elastase and alkaline phosphatase activities but did not have a direct effect on trypsin-like and collagenase activities. The effects of boron on these enzymes have not been described in previous studies, but it is known that peptidic boric acid compounds form tetrahedral borates that covalently bind to the active site of serine proteases such as elastase (8). These compounds are competitive inhibitors, but as the amino acid moiety is essential for their effect, such a mechanism does not seem to explain the effect of boron on elastase observed in our study. In addition, boron might directly modify the enzyme structure, and therefore the activity, by binding to the cis-diol groups of glycosylated protein chains (9, 10). Both alkaline phosphatase and elastase are glycoproteins, and elastase has two potential N-glycosylation sites, one of which carries a carbohydrate moiety (11). Therefore boron binding to sugars could explain the decrease in enzyme activity. However, collagenases, which also have a N-glycosylation site (12), are not inhibited by boric acid. We cannot exclude the fact that bacterial collagenase used in the *in vitro* test behaves differently from human fibroblast collagenase.

Although boron did not have a direct effect on the trypsin-like activity *in vitro*, the protease activity has been found to be increased in living cells (13). Our results confirmed this finding, showing that boron enhances total protease, collagenase, and cathepsin D activities in fibroblasts. This effect of boron could be explained by a stimulation of the secretion of TNFα. It is known that extracellular matrix turnover is influenced by TNFα (14), which is a multifunctional cytokine with a broad spectrum of activities involved in skin wound healing. Found at an increased concentration in early wound fluid (15), TNFα participates in the inflammatory phase of wound healing (16), stimulating the protease activity, particularly collagenase (17, 18, 19), and inhibiting the collagen gene transcription, synthesis (20, 21) and accumulation (18). TACE (TNFα converting enzyme) and a combination of serine proteases, cysteine proteases and metalloproteinases are involved in TNFα processing and release (14).

So by an indirect activation of proteases, boron could favour the release of TNFα stored in the ECM (22) and the rapid generation of highly localized signals by stimulating the ECM degradation. The stimulation of TNFα release by boron would trigger an amplification loop for ECM turnover.

There have been few reports on the action of boron on phosphatases; some studies have been done on the effects of amino-boronic acids on acid phosphatase activity, showing that these compounds prevent the release of this enzyme by protecting the lysosome membrane (23). This mechanism could not be involved in our study because alkaline phosphatase is not a lysosomal enzyme.

The effect of boron on alkaline phosphatase activity *in vitro* could explain the higher concentrations of phosphorylated proteins in cells observed in its presence. However, as we were unable to demonstrate that boron has the same activity *in vivo*, an equally valid hypothesis is that protein phosphorylation is due to kinase activation in relation with cytokine release. Indeed, Benderdour et al. (7) showed that boron stimulates the release of TNFα, and it is also known that TNFα binding to its receptors triggers transduction signaling via kinase activation and phosphorylation of specific proteins (24).

In addition, TNFα is known to increase the production of reactive oxygen species, particularly superoxide anions ($O_2^{\bullet -}$), in various cell lines (25, 26). The cytotoxic mechanisms of TNFα have been directly linked to intracellular oxygen radical production (27, 28, 29).

The generation of mitochondria-derived reactive oxygen species induced by TNFα is a mechanism through which this agent activates the transcription factor NFκB (30), which in turn, activates the transcription of TNFα, thus further propagating the inflammatory response. We therefore measured free radical generation in relation to boric acid concentrations and of culture time. The $O_2^{\bullet 2-}$ concentration decreased and the H_2O_2 concentration increased in function of boric acid concentrations; this could be due to a direct effect of boron on SOD which dismutates $O_2^{\bullet 2-}$ to

$H_2O_2^{\bullet}$. However, time-dependent generation of free radicals did not reach significance. We might assume that boron triggers a transient rise in free radicals sufficient to activate NFκB. This increase could have been neutralized quickly by antioxidant cell defences and therefore not measurable with the method used. In addition, we found no activation of Zn-Cu SOD *in vitro* (data not shown), but TNFα is known to stimulate mainly mitochondrial Mn-SOD (27). Further experiments are necessary to investigate this hypothesis.

In conclusion, boron increases the turnover of the extracellular matrix; it favours the protein phosphorylation (which could result from the activation of receptors by cytokines). The effect of boron may be due to its direct inhibitory effect on the activities of enzymes such as elastase or alkaline phosphatase, although this inhibition is not observed in living cells. This leads to the hypothesis that part of the boron effect on wound healing is not direct, but indirect, via synthesis of cytokines (e.g. TNFα) involved in wound healing, or via generation of free radicals (or other compounds) and activation of transcription factors such as NFκB. It is also noteworthy that TNFα most likely induces the production of VEGF, a strong angiogenic factor (31), and that boron stimulates the production of VEGF (32, 33). This study provides some clues as to the role of boron in wound healing, but further studies are necessary to identify the targets of boron.

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References

1. Blech MF, Martin C, Borrelly J, and Hartemann P (1990) Traitement des plaies profondes avec perte de substance; Intérêt d'une solution d'acide borique à 3%. Presse Médicale 19: 1050–1052
2. Blech MF, Martin C, Pichon C, Borrelly J, and Hartemann P (1990) The clinical and bacteriologic out come of wounds using different local antiseptic. J. Orthop. Surgery B 4: 123–129
3. Litovitz TL, Klein-Schwartz W, Oderda GM, and Schmitz BF (1988) Clinical manifestations of toxicity in a series of 784 boric acid ingestions. Am. J. Emerg. Med. 6: 209–213
4. Linden CH, Hall AH, Kulig KW, and Rumack BH (1986) Acute ingestion of boric acid. J. Toxicol. Clin. Toxicol. 24: 269–279
5. Sobel JD, and Chaim W (1997) Treatment of Torulopsis glabatra vaginitis; Retrospective review of boric acid therapy. Clin. Infect. Dis. 24: 649–652
6. Benderdour M, Hess K, Dzondo-Gadet M, Dousset B, Nabet P, and Belleville F (1997) Effect of boric acid solution on cartilage metabolism. Biochem. Biophys. Res. Comm. 234: 263–268
7. Benderdour M, Hess K, Dzondo-Gadet M, Nabet P, Belleville-Nabet F, and Dousset B (1998) Boron matrix and TNFα synthesis in human fibroblasts. Biochem. Biophys. Res. Commun. 246: 746–751
8. Snow RJ, and Bachovchin WW (1995) Boronic acid inhibitors of dipeptidyl peptidase IV: a new class of immunosuppressive agents. Adv. Med. Chem. 3: 149–177
9. Penn SG, Hu H, Brown PH, and Lebrilla CB (1997) Direct analysis of sugar alcohol borate complexes in plant extracts by matrix-assisted laser desorption/ionization fourier transform mass spectrometry. Anal. Chem. 69: 2471–2477
10. Hu H, Penn SG, Lebrilla CB, and Brown PH (1997) Isolation and characterization of soluble boron complexes in higher plants. The mechanism of phloem mobility of boron. Plant. Physiol. 113: 649–655
11. Wendorf P, Linden D, Sziegoleit A, and Geyer R (1991) Carbohydrate structure of human pancreatic elastase 1. Biochem. J. 278: 505–514
12. Knaüper V, Lopez-Otin C, Smith B, Knight G, and Murphy G (1996) Biochemical characterization of human collagenase-3. J. Biol. Chem. 271: 1544–1550
13. Benderdour M, Hess K, Nzietchueng RM, Nabet P, Belleville F, and Dousset B (1999) Boron and extracellular matrix. In: Abdulla M, Bost M, Gamon S, Arnaud P, Chazot G (Eds) New aspects of Trace Elements research. Smith-Gordon, UK, pp. 17–21
14. Watanabe N, Nakada K, and Kobayashi Y (1998) Processing and release of tumor necrosis factor alpha. Eur. J. Biochem. 253: 576–582
15. Linz DN, Garcia VF, Arya G, and Ziegler MM (1994) Prostaglandin and tumor necrosis factor levels in early wound inflammatory fluid: effects of parenteral omega-3 and omega-6 fatty acid administration. J. Pediatr. Surgery 29: 1065–1070
16. Bettinger DA, Pellicane YV, Wallace CT, Dorne RY, Diegelmann RF, Ronzo L, Cohen IK, and DeMaria EJ (1994) The role of inflammatory cytokines in wound healing: accelerated healing in endotoxin-resistent mice. J. Trauma. 36: 810–814
17. Nelimarkka LO, Nikkari ST, Ravanti LS, Kahari VM, and Jarvelainen HT (1998) Collagenase-1, stromelysin-1 and 92 kDa gelatinase are associated with tumor necrosis factor-alpha induced morphological change of human endothelial cells *in vitro*. Matrix Biol. 17: 293–304
18. Kitzis V, Engrav LH, and Quinn LS (1999) Transient exposure to tumor necrosis factor-alpha inhibits collagen accumulation by cultured hypertrophic scar fibroblasts. J. Surg. Res. 87: 134–141
19. Siwik DA, Chang DL, and Colucci WS (2000) Interleukin-1 beta and tumor necrosis factor-alpha decrease collagen synthesis and increase matrix metalloproteinase activity in cardiac fibroblasts *in vitro*. Circ. Res. 86 (12): 1259–1265
20. Solis-Herruzo JA, Brenner DA, and Chojkier M (1988) Tumor necrosis factor alpha inhibits collagen gene transcription and collagen synthesis in cultured human fibroblasts. J. Biol. Chem. 263: 5841–5845
21. Hernandez I, De La Torre P, Rey-Campos J, Garcia I, Sanchez JA, Munoz R, Rippe RA, Munoz-Yague T, and Solis-Herruzo JA (2000) Collagen alpha1 (I) gene contains an element responsive to tumor necrosis factor-alpha located in the 5' untranslated region of its first exon. DNA Cell Biol. 19: 341–352
22. Taipale J, and Keski-Oja J (1997) Growth factors in the extracellular matrix. FASEB J. 11: 51–59
23. Rajendran KG, Chen SY, Sood A, Spielvogel BF, and Hall IH (1995) The anti-osteoporotic activity of amino-carboxyboranes in rodents. Biomed. Pharmacother 49: 131–140
24. Gueydan C, and Coessens E (1997) Avancées et perspectives de la recherche sur le facteur de nécrose tumorale (TNF). Médecine/Sciences 13: 83–88
25. Hennet T, Richter C, and Peterhans E (1993) Tumor necrosis factor-alpha induces superoxide anion generation in mitochondria of L929 cells. Biochem. J. 289: 587–592

26. Meier B, Radeke HH, Selle S, Younes M, Sies H, Resch K, and Habermehl GG (1989) Human fibroblasts release reactive oxygen species in response to interleukin-1 or tumour necrosis factor-alpha. Biochem J. 263: 539–545
27. Wong GHW, Elwell JH, Oberley LW, and Goeddel DV (1989) Manganous superoxide dismutase is essential for cellular resistance to cytotoxicity of tumor necrosis factor. Cell 58: 923–931
28. Schulze-Osthoff K, Bakker AC, Vanhaesebroeck B, Beyaert R, Jacob WA, and Fiers W (1992) Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. J. Biol. Chem. 267: 5317–5323
29. Hirose K, Longo DL, Oppenheim JJ, and Matsushima K (1993) Overexpression of mitochondrial manganese superoxide dismutase promotes the survival of tumor cells exposed to Interleukin-1, tumor necrosis factor, selected anticancer drugs, and Ionizing radiation. FASEB J. 7: 361–368
30. Shreck R, and Baeuerle KA (1994) Assessing oxygen radicals as mediators In activation of inducible eukariotic transcription factor NF-κB. Methods Enzymol. 234: 151–163
31. Ryuto M, Ono M, Izumi H, Yoshida S, Weich HA, Kohno K, and Kuwano M (1996) Induction of vascular endothelial growth factor by tumor necrosis factor alpha in human glioma cells. Possible roles of SP-1. J. Biol. Chem. 271: 28220–28228
32. Dzondo-Gadet M, Nzietchueng RM, Hess K, Nabet P, Belleville F, and Dousset B (2002) Action of boron at the molecular level: effects on transcription and translation in an acellular system. Biol. Trace. Elem. Res. 85 (1): 23–33
33. Dousset B, Benderdour M, Hess K, Nzietchueng RM, Belleville F, and Duprez A (2000) Effects of boron in wound healing. Experiments on nude mice. In: Roussel AM, Anderson RA, Favier AE (Eds) Trace Elements in Man and Animal (TEMA 10). Kluwer Academic/Plenum Publishers, New York, pp. 1061–1065